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13. ABSTRACT *(Maximum 200 words)*

The polypeptide heregulin has been shown to reverse the transformed phenotype of certain cultured breast cancer cell lines, and hence convert them to more normal, milk-producing cells. In contrast, heregulin is reported to induce proliferation of other breast cancer cell lines. The mechanism by which heregulin alternatively stimulates the proliferation or differentiation of human breast cancer cells was investigated. In the first year of funding, efforts were made to develop assays for quantifying the relative state of differentiation of breast cancer cells. Lipid droplet formation, indicative of the formation of milk by differentiated breast cells, was assayed by light microscopy with the stain Oil Red-O. Production of milk protein was assayed by Western immunoblotting of cell lysates with an antibody recognizing casein. E-cadherin, a cell adhesion molecule expressed at high levels in differentiated cells and low levels in transformed cells, was also detected by Western immunoblotting of breast cancer cell lysates. Although each of these assays should be a valid indicator, we have so far not observed consistent heregulin-dependent changes in the differentiation status of the various breast cancer cell lines examined. Future studies will determine whether specific heregulin-induced intracellular signaling pathways can trigger the differentiation of breast cancer cells.

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(for Morven Sheaver)

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INTRODUCTION

Breast cancer cells have been observed to express abnormally high levels of receptor proteins in the ErbB family, which includes the EGF receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (1-4). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (5). Given that these receptors activate mitogenic signaling pathways, it is possible that they play a role in the abnormal proliferation of breast cancer cells. The polypeptide heregulin (6) is secreted from breast cancer cells (7), and has been shown to activate ErbB2, ErbB3 and ErbB4 receptor proteins (8-11). Whereas ErbB4 can respond to heregulin independently, ErbB2 and ErbB3 have been shown to function together as a coreceptor for heregulin (12). With the discovery of heregulin (originally designated as Neu differentiation factor) came the observation that this factor could induce the re-differentiation of certain cultured breast cancer cell lines, specifically the cell lines MDA-MB-453 and AU-565 (13). Hence, in response to heregulin, these breast cancer cells show a flatter morphology, the presence of lipid droplets, and elevated levels of the milk protein casein. The observation that heregulin can alternatively induce either the proliferation or the re-differentiation of breast cancer cells raises numerous questions about the mechanisms by which this ErbB receptor ligand activates cellular responses. Presumably, clarifying these cellular control mechanisms would lead to a better understanding of breast cancer development, which in turn could lead to the discovery of novel therapeutic or prophylactic measures. Multiple signaling pathways are engaged by ErbB family receptors in response to heregulin. The focus of the proposed research is to identify those signaling pathways that alternatively elicit either the proliferation or differentiation of breast cancer cells.

BODY

In the first year of funding, progress was made towards the development of methods for assaying the relative state of differentiation of cultured breast cancer cells. Two of these methods, Oil Red-O staining of lipid droplets, which represents the production of milk lipids, and casein immunoblotting, which detects the production of milk proteins, have been previously used to study breast cancer cells. A third method, immunoblotting of E-cadherin, detects the expression of a cell adhesion molecule known to be generally expressed in differentiated cells, and attenuated in expression with their cancerous transformation, the loss of cell adhesion being associated with the progressive invasion, migration and metastasis of cancer cells.

Lipid droplets are a relatively obvious characteristic of normal secretory breast epithelial cells, and are present in transformed cells to a greater or lesser extent, depending upon their relative state of de-differentiation. Figure 1 shows typical results of our Oil Red-O staining assay of lipid droplets in cultured cells. As is readily seen in the light microscope, cells in the MDA-MB-468 breast cancer line are relatively differentiated and show abundant lipid droplets. The effect of heregulin on the state of differentiation of various breast cancer cell lines was also examined by use of the Oil Red-O assay. Figures 2 and 3 show the effect of heregulin treatment on two representative cell lines, SK-BR-3 and T47D. To some extent, an eight-day exposure of cells to heregulin enhance the number and density of Oil Red-O-staining lipid droplets. However, we could not demonstrate a statistically significant enhancement of lipid droplet formation in any of the cell lines examined, which included the MDA-MB-453 cell line with which the differentiating effects of heregulin were first observed. Attempts to enhance the differentiating effects of heregulin by variation of culture conditions and heregulin exposure time were also unsuccessful. We concluded that observation of the differentiating effects of heregulin, as previously observed with the MDA-MB-453 cell line, must depend very subtly on cell culture conditions or other undiscovered parameters.

Two other assays of cellular differentiation were also developed. These involved the immunologic detection of β-casein (an abundant milk protein) and E-cadherin (a cell adhesion molecule generally elevated in differentiated cells). While we could readily detect expression of both β-casein and E-cadherin by Western immunoblotting, we could not detect heregulin-

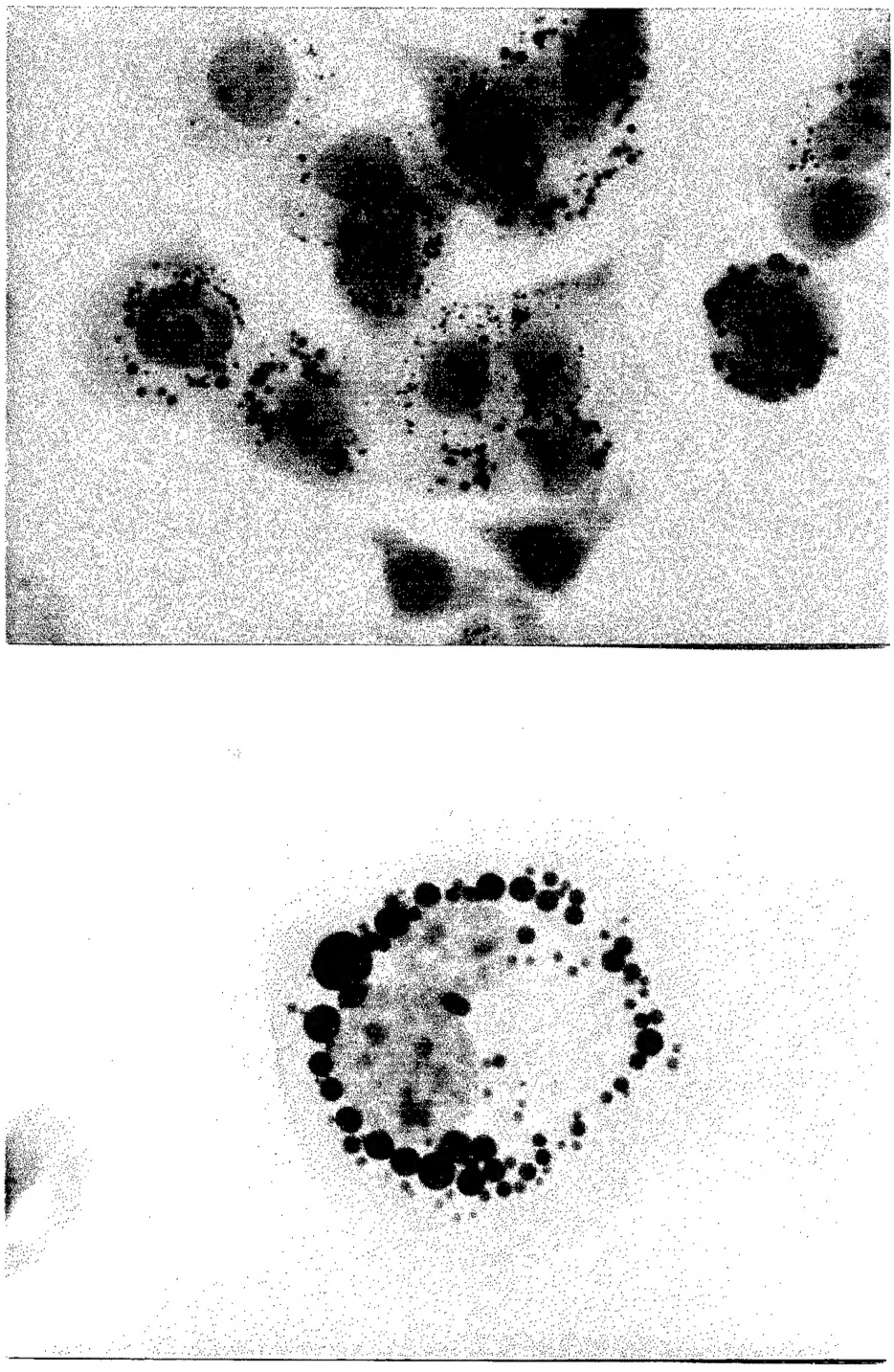

Figure 1. Oil Red-O staining of lipid droplets in the human breast cancer cell line MDA-MB-468. Lipid droplets indicative of the production of milk lipids and the relative state of differentiation of cultured breast cancer cells were stained with the dye Oil Red-O and visualized by light microscopy. Shown are representative images of MDA-MB-468 at cells at 80x (upper panel) and 120x (lower panel) magnification.

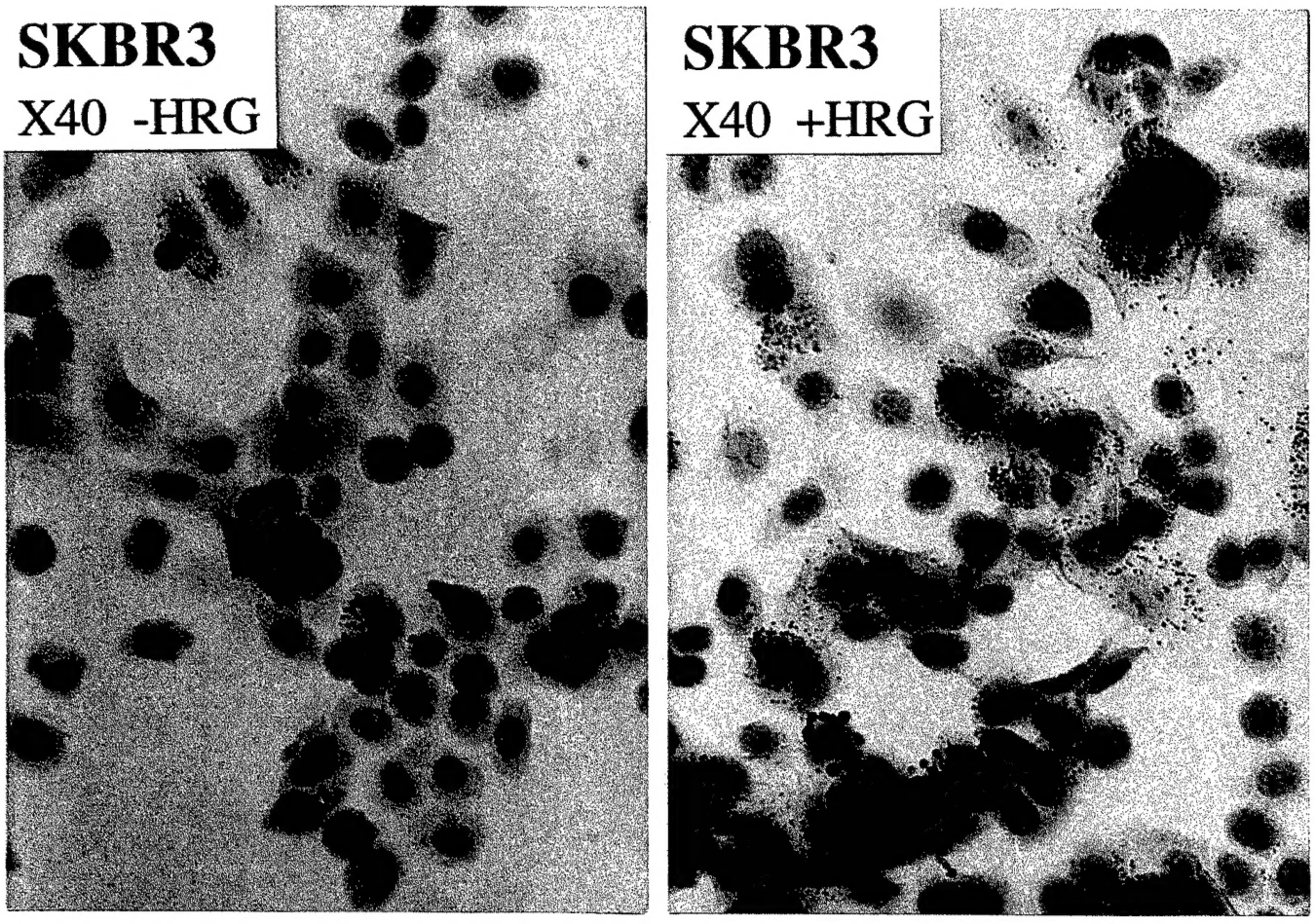

Figure 2. Effect of heregulin treatment on lipid droplet formation in representative human breast cancer cell lines. The SK-BR-3 human breast cancer cell line was cultured for eight days in the presence or absence of 1 nM heregulin-β1 as indicated, after which time the presence of lipid droplets was detected by Oil Red-O staining. Results are representative of several fields of cells examined.

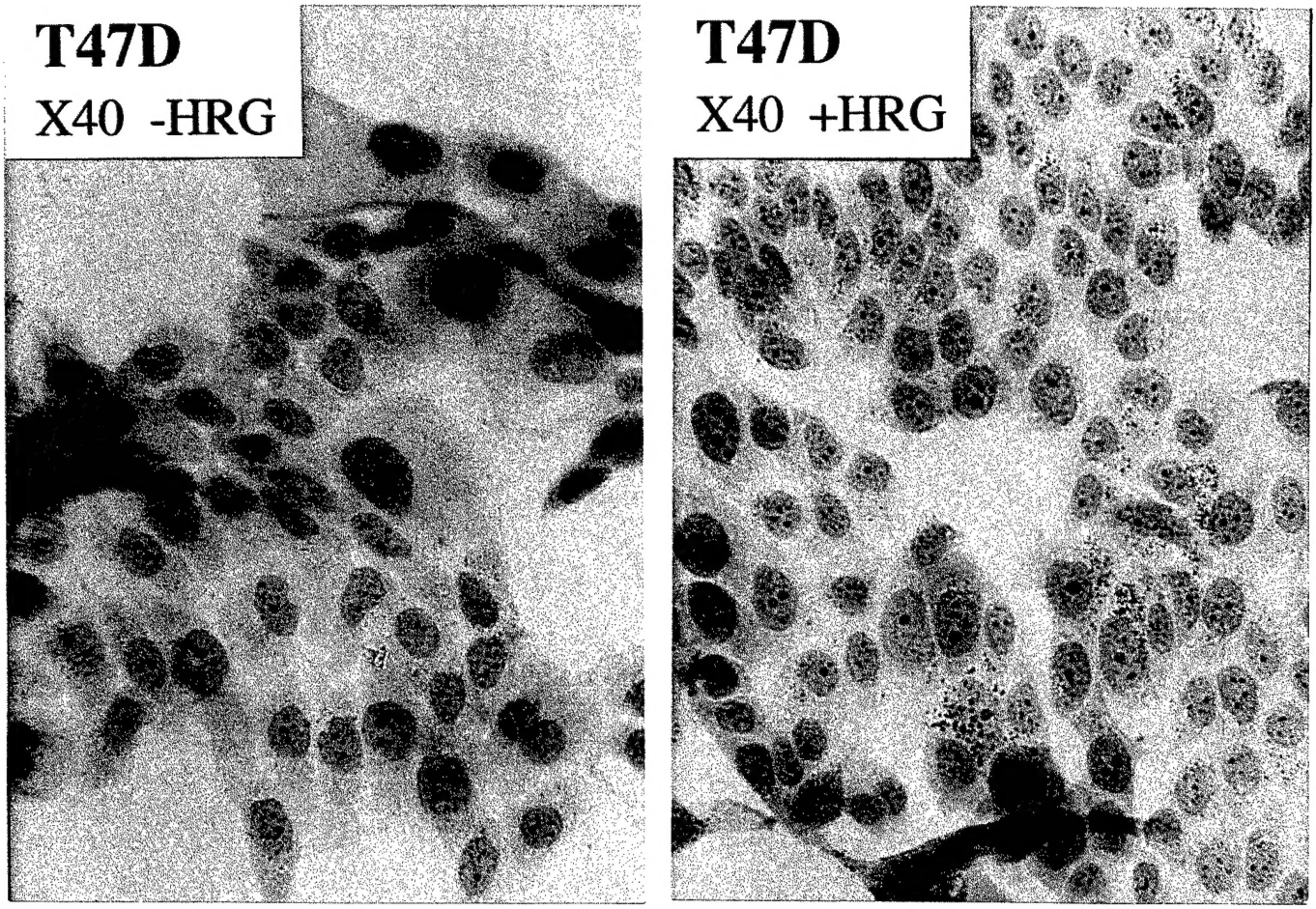

Figure 3. Effect of heregulin treatment on lipid droplet formation in representative human breast cancer cell lines. The T47D human breast cancer cell line was cultured for eight days in the presence or absence of 1 nM heregulin-β1 as indicated, after which time the presence of lipid droplets was detected by Oil Red-O staining. Results are representative of several fields of cells examined.

dependent changes in expression of either differentiation marker. Again, attempts to observe heregulin-dependent changes by variation of cell culture conditions and heregulin exposure time were not effective.

CONCLUSIONS

The first goal of our proposed studies was to develop methods for ascertaining the state of differentiation of cultured breast cancer cells. We have been able to detect three markers of differentiation applicable to breast cancer cells: (1) lipid droplets by Oil Red-O staining (2) β-casein expression; and (3) E-cadherin expression. Mastery of these methods required the investigator to develop facility with light microscopic histochemistry methods and immunologic methods. To date, we have not been able to reproducibly demonstrate the differentiating effects of heregulin previously observed with the MDA-MB-453 cell line. A second cell line in which these effects was observed (AU-565) was not made available to the investigator due to problems of mycoplasm contamination in the stocks of the supplier, but could be the subject of future studies. Given that heregulin has been previously observed to induce differentiation of breast cancer cell lines, we reason that one of the signaling pathways engaged by ErbB family receptors, such as the mitogen-activated protein kinase pathway or the phosphatidylinositol 3-kinase pathway, might be more effective in eliciting differentiation of these cells if activated individually rather than in sum. Hence, future efforts will focus on developing methods for the specific activation of these individual signaling pathways in cultured breast cancer cells. Such methods will include the use of inhibitors to block alternative pathways as described in the original proposal.

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APPENDICES

Key research accomplishments:

1. Development and application of the Oil Red-O histochemical method for detection of lipid droplets in cultured breast cancer cells.
2. Development and application of the Western immunoblotting method for detection of β-casein and E-cadherin expression in cultured breast cancer cells.
3. Investigation of the potential differentiating effects of heregulin treatment on cultured breast cancer cells by assay of markers of cellular differentiation.

Reportable outcomes:

None.

Copies of above cited manuscripts:

Not applicable.